



UNIVERSITI PUTRA MALAYSIA

**QUANTIFICATION OF CIRCULATING EPSTEIN-BARR VIRUS
LATENT MEMBRANE PROTEIN 1 GENE AND ANALYSIS OF 30-BP
DELETION AND *XHO*I-LOSS VARIANTS IN NASOPHARYNGEAL
CARCINOMA**

SEE HUI SHIEN

FPSK(M) 2007 12

**QUANTIFICATION OF CIRCULATING EPSTEIN-BARR VIRUS LATENT
MEMBRANE PROTEIN 1 GENE AND ANALYSIS OF 30-BP DELETION
AND *XHOI*-LOSS VARIANTS IN NASOPHARYNGEAL CARCINOMA**

By

SEE HUI SHIEN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Master of Science**

October 2007



Specially dedicated to,

My parents, sisters and brother,

For their invaluable love, understanding, encouragement and moral support

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment
of the requirement for the degree of Master of Science

**QUANTIFICATION OF CIRCULATING EPSTEIN-BARR VIRUS LATENT
MEMBRANE PROTEIN 1 GENE AND ANALYSIS OF 30-BP DELETION
AND *XHOI*-LOSS VARIANTS IN NASOPHARYNGEAL CARCINOMA**

By

SEE HUI SHIEN

October 2007

Chairman: Professor Seow Heng Fong, PhD

Faculty: Medicine and Health Sciences

Nasopharyngeal carcinoma (NPC) is a human epithelial tumour with a high prevalence in Southern Chinese population. In Malaysia, NPC has become the second most frequent cancers among males and fifth most frequent cancers in females. Genetics, immunologic factors, preserved foods, excessive salts, smoking, various infecting factors are relevant with NPC. A unique feature of NPC is its strong association with Epstein-Barr virus (EBV). Previous studies have shown that EBV-encoded latent membrane protein 1 (LMP1) gene was considered to be associated with the tumourigenesis of NPC. The presence of EBV LMP1 gene variants were shown to be more oncogenic than the LMP1 gene from the prototype virus, B95-8. Free EBV DNA can be detected in serum or plasma from NPC patients and it has been shown to be derived from tumours. This raises the possibility that an easy and non-invasive method may be developed for diagnostic

and disease monitoring purposes in NPC. Thus, the aim of the study is to determine the prevalence of these variants, based mainly on the *XhoI* restriction site polymorphism and the 30bp deletion of LMP1 gene, and to evaluate the potential role of circulating EBV *LMP1* as a molecular marker for diagnosis and disease monitoring in NPC patients.

By employing Polymerase Chain Reaction method (PCR), the presence of 30bp deletion and the loss of *XhoI* restriction site of LMP1 gene in 42 and 10 archival formalin fixed, paraffin-embedded tissues of NPC and non-malignant nasopharyngeal biopsy specimens, respectively, and 35 plasma samples from nasopharyngeal carcinoma were studied. The wild type EBV strain from B95.8 was used as negative control and DNA from 2 NPC tissues as confirmed by DNA sequencing for the presence of 30-bp deletion was used as the positive control in this study. In the quantification of circulating EBV DNA load analysis, 41 plasma samples from NPC patients were used. Standard curve generated by using quantitative Real-Time PCR method against EBV *LMP1* was used to quantify the circulating EBV DNA in 18 NPC subjects at the time of the initial diagnosis, 14 in the middle of treatment and 9 after radiotherapy or chemotherapy. The EBV DNA copy number in 19 apparently healthy adults was also evaluated.

The results showed that: 1) The presence of 30-bp deletion and loss of *XhoI* restriction site can be found in both nasopharyngeal biopsy tissues and also in plasma samples. However, the frequency detected in plasma was lower compared to

primary tumour site. The 30-bp deletion was detected in 55.9% of NPC tissues and 24.1% NPC plasma. Interestingly, 17.2% of plasma samples harboured both the deleted and non-deleted variants, thus, suggestive of dual infections in these patients. The loss of *XhoI* restriction site in LMP1 gene was found in 87.2 % of the NPC tissues and 36.7% of plasma samples. There was no 30-bp deletion and *XhoI*-loss in non-malignant nasopharyngeal tissues. Majority of our samples (59.4% of NPC tissues and 26.9% of plasma samples) showed the presence of both of the 30-bp deletion and the loss of *XhoI* restriction site, which resembles the CAO, C1510, China 1 and DV2 isolated from high endemic area for NPC. 2) The 30-bp deletion and loss of *XhoI* restriction site have been found to be more prevalent in Chinese compared to Malay (30bp-deletion, $p=0.000$; *XhoI*-loss, $p=0.046$), and its percentage were higher in type III (undifferentiated carcinoma) than in type I (squamous cell carcinoma) NPC biopsy tissues (30bp-deletion, $p=0.011$; *XhoI*-loss, $p=0.006$). 3) The EBV DNA detection rate in the plasma of NPC patients (94.4%) was significantly higher than in apparently healthy adults (AHAs) (15.8%). According to the receiver-operating characteristic (ROC) curve analysis, plasma EBV DNA levels at the cut-off of 0 copy/ml combined a sensitivity of 94.4% (C.I.95%=72.6-99.1) with a specificity of 84.2% (C.I.95%=60.4-96.4) for detection of NPC, and a ROC AUC of 0.904 (C.I.95%: from 0.760-0.975). The mean circulating EBV DNA load in the plasma of untreated NPC patients (median=2471 copies/ml) was higher than AHAs (median=0 copy/ml). A significant decrease in EBV load was observed in patients who had undergone radiotherapy (median=0 copy/ml) while three patients had remaining EBV load. The mean of the

post-treatment EBV DNA levels were not statistically different with the AHAs samples. 4) None of the clinicopathological features were associated with the pre-treatment plasma EBV DNA load including tumour histological type and clinical stage.

The important findings in this study are: 1) High frequency of 30-bp deletion and *XhoI*-loss in the *LMP1* gene is present in Malaysian NPC population. The distribution of higher level of 30-bp deletion and *XhoI*-loss in Chinese and Type III NPC may be associated with geographical/ethnic and clinical background. It suggested that these variants may have unique functional properties, which determine disease association or development. 2) The circulating EBV *LMP1* was detectable in NPC patients and it was shown to be proportionally related to the presence of malignant disease, suggested that the *LMP1* may serve as a molecular marker for diagnosis and disease monitoring in NPC.

In conclusion, a high prevalence of 30-bp deletion and *XhoI*-loss in *LMP1* were present in Malaysian NPC. By using the sensitive, accurate and robust real-time PCR technique, we have showed the clinical significance of detecting the EBV *LMP1* in the plasma where the quantification of EBV *LMP1* may be a useful indicator for screening, diagnosis and disease monitoring in NPC.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**KUANTIFIKASI PEREDARAN GEN LATENT MEMBRANE PROTEIN 1
VIRUS EPSTEIN-BARR DAN ANALISIS VARIAN PEMOTONGAN 30-BP
SERTA KEHILANGAN *XHOI* DALAM KANSER NASOFARINKS**

Oleh

SEE HUI SHIEN

Oktober 2007

Pengerusi: Profesor Seow Heng Fong, PhD

Fakulti: Perubatan dan Sains Kesihatan

Kanser nasofarinks (NPC) merupakan sejenis tumor epitelia manusia yang tersebar luas dalam populasi Cina Selatan. NPC merupakan barah yang kedua paling kerap di kalangan lelaki dan kelima paling kerap di kalangan wanita. Genetik, faktor imunologi, makanan pengawet, kelebihan garam, merokok dan pelbagai faktor infeksi adalah berkaitan dengan NPC. Salah satu sifat unik NPC adalah perkaitannya yang kuat dengan virus Epstein-Barr (EBV). Kajian terdahulu telah membuktikan gen latent membrane protein 1 (LMP1) yang dikodkan oleh EBV adalah berkaitan dengan asal usul genesis tumor NPC. Kehadiran varian gen LMP1 EBV telah dibuktikan lebih onkogenik berbanding dengan gen LMP1 dari virus prototaip, B95-8. DNA bebas EBV boleh dikesan di serum atau plasma dari pesakit NPC dan ia telah ditunjukkan berasal dari tumor. Ini telah membangkitkan kemungkinan bahawa sejenis cara yang mudah dan tidak invasif boleh dimajukan

untuk tujuan diagnosis dan pemantauan penyakit dalam NPC. Maka, tujuan kajian kami adalah untuk menentukan penyebaran jenis varian, berdasarkan kepelbagaian dalam tempat pemotongan *XhoI* dan juga pembuangan 30-bp daripada gen *LMP1* serta menilai peranan potensi daripada peredaran EBV *LMP1* sebagai tanda molekular untuk diagnosis dan pemantauan penyakit dalam pesakit NPC.

Dengan menggunakan teknik Polymerase Chain Reaction (PCR), kehadiran pembuangan 30-bp dan kehilangan tempat pemotongan *XhoI* dalam gen *LMP1* dari 42 dan 10 spesimen biopsi NPC dan nasofarinks yang tak malignan, masing masing serta 35 sampel plasma dari NPC telah dikaji. Dalam kajian ini, EBV jenis liar, B95.8 telah digunakan sebagai kontrol negatif dan NPC 1 serta 2 yang dikaji oleh penjujukan DNA untuk kehadiran pembuangan 30-bp telah digunakan sebagai kontrol positif. Empat puluh satu sampel plasma dari pesakit NPC telah digunakan dalam analisis kuantifikasi peredaran DNA EBV. Lengkung piawai yang dihasilkan dengan menggunakan cara kuantitasi Real-Time PCR tentang EBV *LMP1* telah digunakan untuk kuantitasi peredaran DNA EBV dalam 18 subjek NPC pada masa diagnosis, 14 pada pertengahan rawatan dan 9 selepas radioterapi atau kemoterapi. Bilangan DNA dalam 19 dewasa nampak sihat (AHA) telah dinilai.

Keputusan menunjukkan bahawa: 1) Kehadiran pembuangan 30-bp dan kehilangan tempat pemotongan *XhoI* boleh dijumpai dalam kedua-dua tisu biopsi nasofarinks dan juga sampel plasma. Walau bagaimanapun, frekuensi pengesanan di plasma adalah lebih rendah berbanding di tempat tumor utama. Pembuangan 30-bp telah

dikesan dalam 55.9% tisu NPC dan 24.1% plasma NPC. Dengan menariknya, 17.2% sampel plasma mempunyai kedua-dua varian pembuangan dan tanpa pembuangan, mencadangkan infeksi dua kali dalam pesakit ini. Kehilangan tempat pemotongan *XhoI* dalam gen LMP1 telah dijumpai dalam 87.2% tisu NPC dan 36.7% sampel plasma. Tiada pembuangan 30-bp dan kehilangan tempat pemotongan *XhoI* dijumpai dalam tisu nasofarinks tak malignan. Kebanyakan sampel kami (59.4% tisu NPC dan 26.9% sampel plasma) menunjukkan kehadiran kedua-dua pembuangan 30-bp dan kehilangan tempat pemotongan *XhoI* yang mirip CAO, C1510, China 1 dan DV2 yang diasingkan dari kawasan endemik tinggi untuk NPC. 2) Pembuangan 30-bp dan kehilangan tempat pemotongan *XhoI* telah dikesan lebih tersebar di kalangan kaum Cina berbanding dengan Melayu. (pemotongan 30-bp, $p=0.000$; kehilangan *XhoI*, $p=0.046$), dan peratusnya adalah lebih tinggi dalam Jenis III (karsinoma tak membeza) berbanding dengan Jenis I (karsinoma sel skuamus) tisu biopsi NPC (pembuangan 30-bp, $p=0.011$; kehilangan *XhoI*, $p=0.006$). 3) Kadar pengesanan DNA EBV dalam plasma pesakit NPC (94.4%) adalah lebih tinggi berbanding dengan AHA (15.8%). Menurut analisis lengkung receiver-operating characteristic (ROC), tahap DNA EBV plasma pada titik pemotongan 0 copy/ml bergabung dengan 94.4% sensitiviti (C.I.95%=72.6-99.1) dan 84.2% spesifikiti (C.I.95%=60.4-96.4) untuk pengesanan NPC, dengan 0.904 ROC AUC (C.I.95%: dari 0.760-0.975). Min peredaran beban DNA EBV dalam plasma pesakit NPC yang belum terima rawatan (median=2471 copies/ml) adalah lebih tinggi berbanding dengan AHA (median=0 copy/ml). Pengurangan yang ketara dalam beban EBV telah diperhatikan dalam pesakit yang

menjalani radioterapi (median=0 copy/ml) manakala tiga orang pesakit masih mempunyai beban EBV. Min tahap EBV selepas rawatan adalah tidak berbeza secara statistik dengan sampel AHA. 4) Tiada kaitan antara ciri-ciri klinikopatologi dengan beban DNA EBV pada plasma sebelum rawatan termasuk jenis histologi barah dan peringkat klinikal.

Penemuan yang penting dalam kajian ini adalah: 1) Frekuensi yang tinggi dalam pembuangan 30-bp dan kehilangan *XhoI* dalam gen *LMP1* hadir di kalangan populasi NPC Malaysia. Pembahagian tahap yang tinggi untuk pembuangan 30-bp dan kehilangan *XhoI* di kalangan Cina dan Jenis III NPC mungkin berkaitan dengan geografi/etnik dan latar belakang klinikal. Ia telah mencadangkan varian-varian ini mungkin memiliki fungsi yang unik dalam menentukan kehadiran atau pembangunan penyakit. 2) Peredaran EBV *LMP1* telah dikesan di kalangan pesakit NPC dan ia telah ditunjukkan berkaitan secara setimpal dengan kehadiran penyakit malignan, mencadangkan bahawa *LMP1* mungkin berpotensi sebagai tanda molekular untuk diagnosis dan pemantauan penyakit dalam NPC.

Kesimpulannya, penyebaran luas pembuangan 30-bp dan kehilangan *XhoI* hadir di NPC Malaysia. Dengan menggunakan teknik real-time PCR yang sensitive, tepat, dan kuat, kami telah menunjukkan kepentingan klinikal dengan mengesan EBV *LMP1* dalam plasma di mana kuantifikasi EBV *LMP1* boleh dijadikan indikasi yang berguna untuk penyaringan, diagnosis dan pemantauan panyakit dalam NPC.

ACKNOWLEDGEMENTS

First and foremost, I would like to take this opportunity to express my greatest gratitude and appreciation to my supervisor, Prof. Dr. Seow Heng Fong for her valuable guidance, advice, suggestions and endless help during the time I carried out this project. Her careful reviews and constructive criticism have been crucially important for the success of this project as well as the writing of this thesis.

My sincere gratitude is also accorded to my co-supervisor, Dr. Yap Yoke Yeow, for his ever willing to lend his advice and support. His kindness and guidance is very much appreciated.

My sincere thanks go to Dr. Maha Abdullah, Leong Pooi Pooi, Lim Pei Ching, Cheah Hween Yee, Loh Hui Woon and Yip Wai Kien for their valuable guidance and advice. Thank you for being caring and always ready to assist me when I need it most.

Not forgetting also a million thanks to all the members of the Immunology laboratory, Dr. Sharmili, Dr. Rajesh, Choo Chee Wei, Jee Jap Meng, Masriana Hassan, Ooi Suek Chin, Leslie Than, Vincent Leong, Lim Chooi Ling, Koh Rhun Yian, Siti Aishah, Siti Hasrizan, Jervis Chow, Jasmine Lim, Mahathir, and Mr. Anthonysamy for their collaboration, endless support, understanding, and

friendship. I am also grateful to Low Lee Yean, Crystal Lim and Phelim Yong for their generous assistance.

Last but not least, to my parents, sisters and brother, thank you for your endless prayer and support. Collectively, you are my inspiration.

Thank you.

I certify that an Examination Committee has met on 22nd October 2007 to conduct the final examination of See Hui Shien on her Master of Science thesis entitled “Quantification of Circulating Epstein-Barr Virus Latent Membrane Protein 1 Gene and Analysis of 30-BP Deletion and *Xho*I-Loss Variants in Nasopharyngeal Carcinoma” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Examination Committee were as follows:

Mirnalini Kandiah, PhD

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Zamberi Sekawi, PhD

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Fauziah Othman, PhD

Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Sam Choon Kook, PhD

Lecturer
National Institute of Education
Nanyang Technological University
(External Examiner)

HASANAH MOHD. GHAZALI, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 17 December 2007

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Seow Heng Fong, PhD

Professor

Faculty of Medicine and Health Sciences

Universiti Putra Malaysia

(Chairman)

Yap Yoke Yeow, M.Med.

Lecturer and Otorhinolaryngologist

Faculty of Medicine and Health Sciences

Universiti Putra Malaysia

(Member)

AINI IDERIS, PhD

Professor and Dean

School of Graduate Studies

Universiti Putra Malaysia

Date: 22 January 2008

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

SEE HUI SHIEN

Date:

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vii
ACKNOWLEDGEMENTS	xi
APPROVAL	xiii
DECLARATION	xv
LIST OF TABLES	xix
LIST OF FIGURES	xxi
LIST OF ABBREVIATIONS	xxiv
 CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	5
2.1 Nasopharyngeal carcinoma (NPC)	5
2.1.1 Anatomy of the nasopharynx	5
2.1.2 Pathology of NPC	6
2.1.3 Epidemiology	8
2.1.4 Risk factor and etiology	9
2.1.5 Clinical presentation, diagnosis and staging	13
2.1.6 Treatment	14
2.1.7 Difficulties in diagnosis, treatment response monitoring, prognosis and progression	16
2.2 Epstein-Barr virus (EBV)	20
2.2.1 EBV biology and immunology	20
2.2.2 Primary and persistent EBV infection	24
2.3 Epstein-Barr virus (EBV) and nasopharyngeal carcinoma (NPC)	27
2.3.1 EBV infection in NPC	27
2.3.2 EBV gene expression in NPC	30
2.3.3 Latent Membrane Protein 1 (LMP1) variant and its transformation potential in NPC	35
2.3.4 Circulating EBV DNA in plasma	37
2.4 Quantitative Real-Time Polymerase Chain Reaction (Quantitative Real-Time PCR)	40
3 MATERIALS AND METHODS	44
3.1 Specimen collection	44

3.1.1	Tissue specimens	44
3.1.2	Blood	44
3.2	Cell culture	45
3.3	DNA extraction	45
3.3.1	Tissue specimens	45
3.3.2	Plasma samples	46
3.3.3	Cultured cells	47
3.4	PCR amplification	48
3.4.1	PCR reagents	48
3.4.2	PCR amplification of <i>Latent Membrane Protein 1</i> (<i>LMP1</i>) 30-base pair deletion region	49
3.4.3	PCR amplification of <i>XhoI</i> restriction site	49
3.4.4	PCR amplification of B-actin gene	50
3.5	PCR product purification	52
3.6	<i>XhoI</i> restriction endonuclease digestion of PCR product	52
3.7	EBV DNA quantification	53
3.7.1	Plasmid clones	53
3.7.2	Preparation of chemically competent <i>E. coli</i> cells	53
3.7.3	Heat shock transformation method	54
3.7.4	Miniprep of plasmid DNA	55
3.7.5	DNA sequencing	56
3.7.6	<i>Bam</i> HI restriction endonuclease digestion of plasmid DNA	56
3.7.7	Gel purification of plasmid DNA	57
3.7.8	Standard curve generation	58
3.7.9	Real time PCR quantification of EBV LMP1 gene	59
3.8	Statistical Analysis	60
4	ANALYSIS OF THE EBV LMP1 VARIANTS IN NASOPHARYNGEAL CARCINOMA	61
4.1	Introduction	61
4.2	Clinicopathological data of patients	63
4.3	Detection of the EBV LMP1 gene 30-bp deletion	66
4.3.1	Result	66
4.3.2	Discussion	76
4.4	Detection of the <i>XhoI</i> polymorphism in the exon 1 of LMP1 gene	82
4.4.1	Result	82
4.4.2	Discussion	89
4.5	Summary of detection of the 30-bp deletion in exon 3 and <i>XhoI</i> polymorphism in exon 1 of <i>LMP1</i> in NPC	93
4.5.1	Results	93
4.5.2	Discussion	94
4.6	Conclusion	97

5	QUANTIFICATION OF EBV DNA LOAD IN NASOPHARYNGEAL CARCINOMA	98
5.1	Introduction	98
5.2	Results	100
5.2.1	Clinicopathological data of NPC patients	100
5.2.2	Extracted plasma DNA	102
5.2.3	The standard curve	103
5.2.3.1	Plasmid DNA	103
5.2.3.2	Inter-assay reproducibility	110
5.2.4	EBV DNA load between NPC patients and apparently healthy adults	112
5.2.5	Correlation between plasma NPC EBV DNA levels and clinicopathological characteristics of NPC patients	127
5.3	Discussion	129
6	CONCLUSIONS AND FUTURE RECOMMENDATIONS	134
6.1	Conclusions	134
6.2	Future recommendations	137
	REFERENCES	138
	APPENDICES	162
	BIODATA OF THE AUTHOR	173

LIST OF TABLES

Table		Page
2.1	The tumor, node, metastasis (TNM) classification of the American Joint Committee (AJCC) on Cancer	18
2.2	Stage grouping of NPC by AJCC	19
2.3	Patterns of latent gene expression in EBV-related malignancies	21
3.1	Target genes, primers and nucleotide sequences.	51
4.1	Clinicopathological details of NPC patients for the NPC tissues specimens used in the analysis of LMP1 gene variants.	64
4.2	Clinicopathological details of NPC patients for the plasma samples used in the analysis of LMP1 gene variants.	65
4.3	30-bp Deletion in NPC and non-malignant nasopharyngeal tissues	68
4.4 (A)	Sequence variation in the <i>LMP1</i> Exon 3 in isolates from two nasopharyngeal carcinoma tissues	69
4.4 (B)	Comparison of deduced amino acid sequences in the <i>LMP1</i> exon 3 in isolates from two nasopharyngeal carcinoma tissues	70
4.5	Association between clinicopathological data with 30-bp deletion studies in NPC tissue	71
4.6	30-bp deletion in NPC plasma samples	74
4.7	Association between clinicopathological data with 30-bp deletion studies in NPC plasma	75
4.8	<i>XhoI</i> polymorphism in NPC and non-malignant nasopharyngeal tissues	83



4.9	Association between clinicopathological data with <i>XhoI</i> polymorphism in NPC tissue	84
4.10	<i>XhoI</i> polymorphism in NPC plasma samples	87
4.11	Association between clinicopathological data with <i>XhoI</i> polymorphism in NPC plasma	88
4.12	Comparison between the prevalence of the 30-bp deletion and <i>XhoI</i> loss variants in NPC tissues and plasma	93
5.1	Clinicopathological data of NPC patients for the plasma samples used in the quantification of EBV <i>LMP1</i> in NPC	101
5.2	C _T values (mean \pm Standard Deviation) from five replicates amplification	111
5.3	EBV DNA load in nasopharyngeal carcinoma (NPC) patients	119
5.4	EBV DNA load in Apparently Healthy Adults (AHA)	120
5.5	Sensitivity, specificity and predictive values of plasma EBV DNA load for identification of NPC. Numbers in square brackets refer to lower and upper 95% confidence intervals (CI).	122
5.6	Correlation between clinicopathological characteristics with EBV DNA load in NPC plasma.	128

LIST OF FIGURES

Figure		Page
2.1	The nasopharynx is the upper part of the pharynx behind the nose.	6
2.2	The Epstein-Barr virus (EBV) genome.	24
2.3	A model for Epstein–Barr virus (EBV) infection and persistence.	26
2.4	Protein sequence comparison of BLMP1 and NLMP1.	36
4.1	Agarose electrophoresis gel of the LMP1 gene exon 3 amplicons with and without the 30-bp deletion from tissues samples as represented by the 156-bp and 186-bp band, respectively.	68
4.2	Agarose electrophoresis gel of the LMP1 gene exon 3 amplicons with and without the 30-bp deletion from plasma samples as represented by the 156-bp and 186-bp band, respectively.	73
4.3	Representative gel electrophoresis of <i>LMP1</i> amplicons from NPC plasma showing simultaneous presence of wild type and 30-bp deletion variants.	74
4.4	PCR amplification of <i>LMP1</i> exon 1 covering the <i>XhoI</i> restriction site. The PCR products shown were before purification.	85
4.5	Purified PCR products of <i>LMP1</i> N-terminal covering the <i>XhoI</i> restriction site.	85
4.6	<i>XhoI</i> digestion analyses of NPC and non-malignant tissues as well as plasma samples from NPC patients.	86
5.1	Representative electrophoresis gel of the PCR amplification of β -actin gene with extracted DNA from plasma of NPC patients and apparently healthy adult.	102

5.2	Representative electrophoresis gel of the purified p-TOPO <i>LMP1</i> plasmid DNA	105
5.3	Representative electrophoresis gel of the PCR amplification of 447bp <i>LMP1</i> gene from extracted plasmid DNA.	105
5.4	BLAST result of the sequencing sequence against Epstein-Barr virus genome, strain B95.8 (GenBank Accession no.V01555).	106
5.5	Representative electrophoresis gel of the recombinant p-TOPO <i>LMP1</i> plasmid with <i>Bam</i> HI restriction enzyme digestion.	107
5.6	Representative electrophoresis gel of the PCR amplification of <i>LMP1</i> gene with purified <i>Bam</i> HI digested p-TOPO <i>LMP1</i> plasmid DNA	107
5.7 (A)	Representative real time PCR cycling profiles of <i>LMP1</i> from the p-TOPO <i>LMP1</i> plasmid DNA with starting EBV DNA ranging from 74.8 to 7480000 copies.	108
5.7 (B)	Representative standard curve for <i>LMP1</i> PCR generated from six serial dilutions of the p-TOPO <i>LMP1</i> standard against their respective threshold value (C_T)	108
5.7 (C)	Representative melting curves for <i>LMP1</i> amplification products from p-TOPO <i>LMP1</i> plasmid DNA of six concentrations.	109
5.8	Scatter plot of inter-assay comparisons between the six different concentration of EBV copies number and C_T values.	110
5.9(A)	Representative real time PCR cycling profiles of <i>LMP1</i> from the p-TOPO <i>LMP1</i> plasmid DNA with starting EBV DNA ranging from 74.8 to 7480000 copies and the NPC samples.	116
5.9 (B)	Representative standard curve for <i>LMP1</i> PCR generated from six serial dilutions (indicated in blue dots) of the p-TOPO <i>LMP1</i> standard against their respective threshold value (C_T).	116

5.9(C)	Representative melting curves for <i>LMPI</i> amplification products from p-TOPO <i>LMPI</i> plasmid DNA of six concentrations and NPC samples.	117
5.10	Data with the cycle threshold (C_T) value of the amplification plot of pooled plasma of NPC individuals co-extracted with each samples. The coefficient of variation was 1.01% (mean $C_T=29.56 \pm 0.30$).	118
5.11	Box plots represent the plasma EBV DNA concentrations in nasopharyngeal carcinoma (NPC) patients and apparently healthy adults (AHA).	121
5.12	Receiver-operating characteristic (ROC) curve analysis of plasma EBV DNA for the prediction of NPC.	122
5.13	Plasma cell-free EBV DNA in pre-treatment and intra-treatment NPC patients.	123
5.14	Plasma cell-free EBV DNA in untreated and treated NPC patients.	124
5.15	Box plots represent the plasma EBV DNA concentrations in pre-, intra-, and post-treatment in nasopharyngeal carcinoma (NPC) patients.	125
5.16 (A)	Representative real time PCR cycling profiles of <i>LMPI</i> from the p-TOPO <i>LMPI</i> plasmid DNA with starting EBV DNA ranging from 74.8 to 7480000 copies, normal adults PBMC, TW06 and CNE1.	126
5.16 (B)	Melting curves for <i>LMPI</i> amplification products from p-TOPO <i>LMPI</i> plasmid DNA of six concentrations, normal adults PBMC, TW06 and CNE1.	127

LIST OF ABBREVIATIONS

~	Approximately
bp	Base-pair
kb	Kilobase-pair
β	Beta
°C	Degree of Celsius
%	Percentage
μl	Microliter
μm	Micrometer
ml	Milliliter
Mg	Miligram
μg	Microgram
ng	nanogram
A	Absorbance
AHA	Apparently healthy adults
AIDS	Acquired Immune Deficiency Syndrome
AJCC	American Joint Committee on Cancer
AKT	v-akt murine thymoma viral oncogene homolog
AML	Acute myelogenous leukemia
ANOVA	Analysis of Variance
AUC	Area under the ROC curve
<i>BamH</i>	<i>Bacillus amyloliquefaciens</i> H